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Interspecific Hybridization between *Arisaema sikokianum* and *A. tosaense* (Araceae) Confirmed through Nuclear and Chloroplast DNA Comparisons

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Morphologically intermediate plants between *Arisaema sikokianum* Franch. & Sav. and *A. tosaense* Makino were newly found in Tokushima Prefecture, Shikoku, Japan. Molecular analysis using PCR-RFLP of internal transcribed spacer (ITS) in nuclear DNA (nrDNA) indicated that these putative hybrids have a combined pattern of the two putative parental species. Moreover, the sequence results of chloroplast DNA (cpDNA) analysis of the putative hybrids were on the whole identical with both *A. sikokianum* or *A. tosaense*. The results suggest that the plants are hybrids between *A. sikokianum* and *A. tosaense* and that they have exchanged genes with the two parental species.

Key words: Araceae, *Arisaema*, *Arisaema sikokianum*, *Arisaema tosaense*, interactive gene exchange, ITS, molecular analysis, PCR-RFLP, *trn*

The genus *Arisaema* Martius (Araceae), which has a large, often colored and conspicuous bract (spathe), subtending and enveloping a bisexual or unisexual spadix with numerous small flowers, comprises approx. 25 species in Japan (Ohashi 1982). Within the Japanese Archipelago, Shikoku is remarkable in harboring many endemic and semiendemic species of the genus. Among them, *Arisaema sikokianum* Franch. & Sav. is characteristic. It has a conspicuous spathe, smoky purple at the base, a snow white cup and a large hood with purple, green and white stripes and leaves with three to five leaflets (Fig. 1A, See detail Table 1). *Arisaema tosaense* Makino has a characteristic green spathe extending to approx. 30–45 cm before it bends downward to the ground and has leaves with 7 to 11 leaflets (Fig. 1B, Table 1). Because there are clear morphological distinctions between those two species, they can be distinguished easily in the field even when growing together. Both species occur in Shikoku and adjacent areas of Japan (Ohashi 1982). In Kochi Prefecture, *A. sikokianum* and *A. tosaense* grow sympatrically in shaded forests, but the habitat of each is a little different. We noted that *A. tosaense* occurs with *A. sikokianum* or at higher elevations than *A. sikokianum*. The flowering phenology of the two species overlaps slightly in the mountains (Table 1). Ohashi (1982) and Kobayashi (2009) reported interspecific hybrids between these two

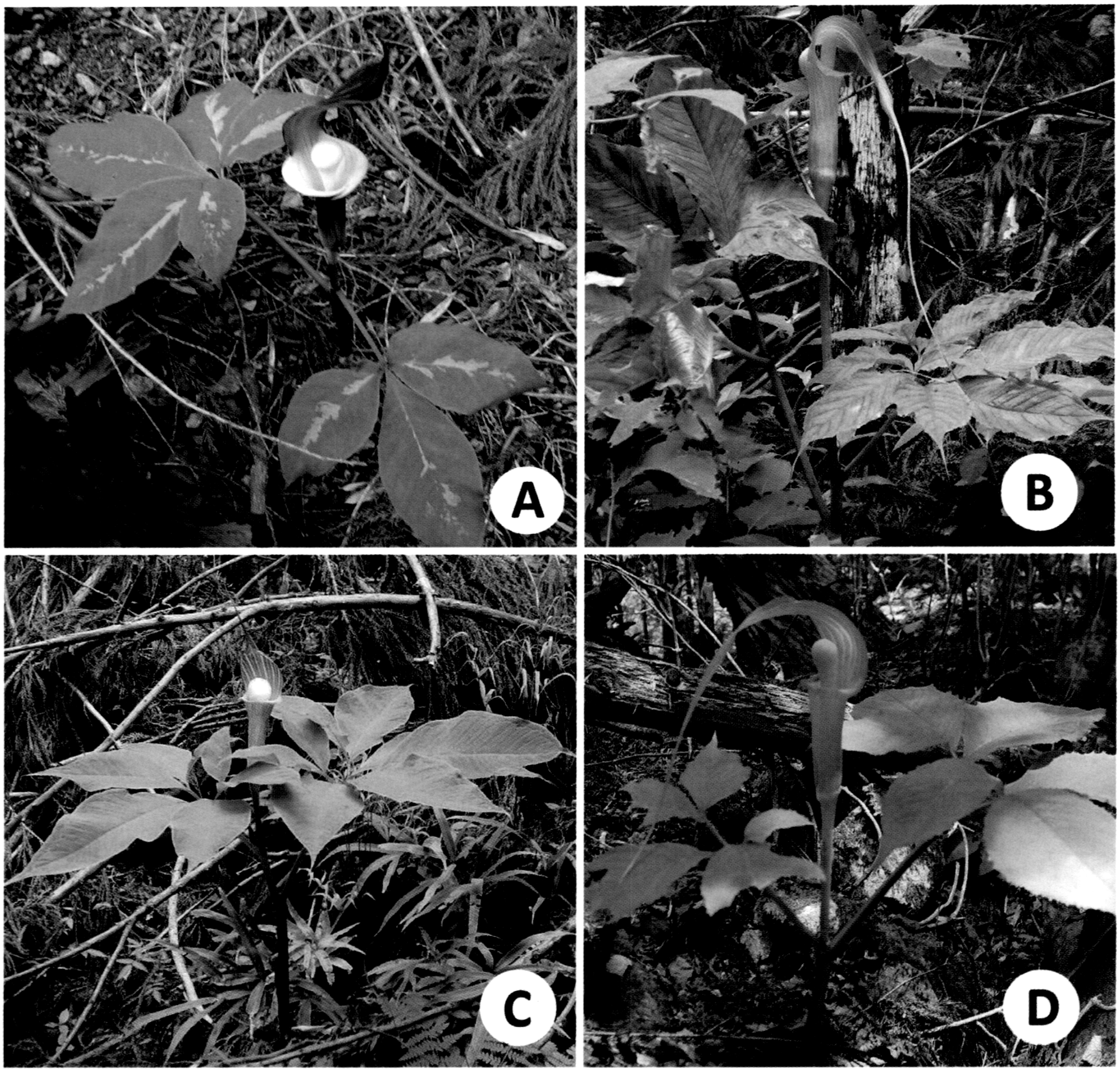


FIG. 1. Species of *Arisaema* examined in this study. A: *A. sikokianum* (sample No. 1). B: *A. tosaense* (sample No. 8). C: putative hybrid (sample No. 5). D: putative hybrid (sample No. 7). Sample numbers corresponded to numbers in Table 2.

TABLE 1. Morphological characteristics of sample used in this study.

Trait	*A. sikokianum*	Putative hybrid			*A. tosaense*
		I	II	III	
Leaf Characteristics					
Leaflets	3 to 5	9	7	7	7 to 11
Reproductive Characteristics					
Spathe Tip Direction	Upward	Upward	Drooping	Drooping	Drooping
Spathe Color	Purple	Purple	Green	Green	Green
Appendage Shape	Capitate	Capitate	Cylindrical	Capitate	Cylindrical
Appendage Color	White	White	Green	Green	Green
Flowering Phenology	April to May	Early June	Early June	Early June	Late May to June

species only in Kochi Prefecture. The area of sympatry of the two species, however, extends over a wide range in Shikoku, but hybridization in other areas has not been reported until now, where we discuss putative hybrids of *A. sikokianum* and *A. tosaense* (Fig. 1 D) in Tokushima Prefecture, Shikoku (Fig. 2III) as well as at a locality (Fig. 2II) previously reported by Murata (1962). To clarify maternal and paternal parents of the putative hybrid, we conducted molecular analyses using nuclear DNA (nrDNA) and chloroplast DNA (cpDNA) sequences. Our findings in hybrids at localities different from those reported previously suggests that extensive hybridization may occur between these two species.

Materials and Methods

Morphologically intermediate plants between *Arisaema sikokianum* and *A. tosaense* were found at three localities shown in Fig. 2, two of them in Kochi Prefecture and one in Tokushima Prefecture (Table 2). All of the hybrids were found with *A. sikokianum* and *A. tosaense*. Voucher specimens are deposited in the herbarium of Makino Botanical Garden, Kochi (MBK).

For the molecular analyses, total DNA was isolated from 200–300 mg of leaves using a Plant

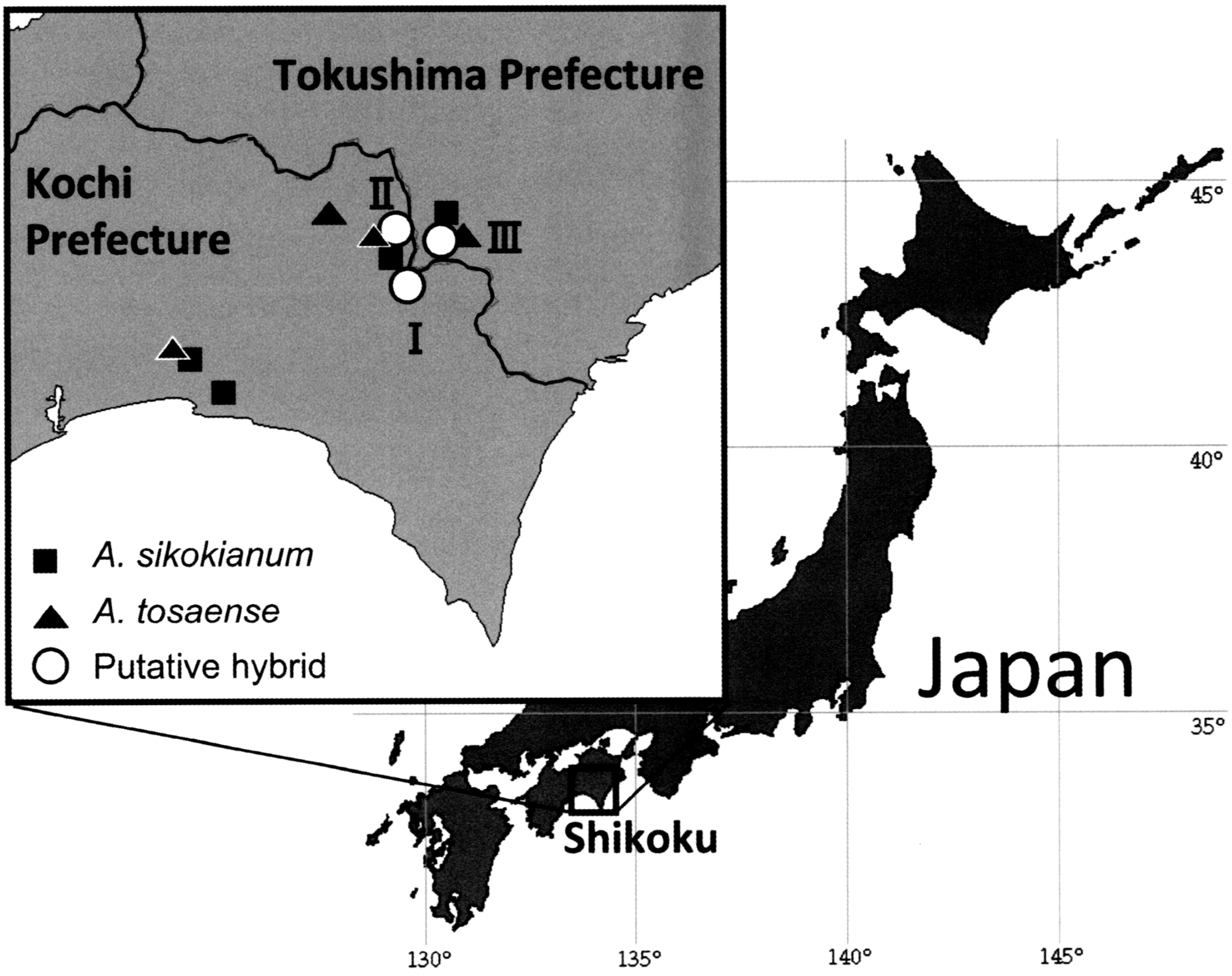


FIG. 2. Sampling localities of putative hybrids of *Arisaema sikokianum* and *A. tosaense*. I: Kochi Pref., Aki City, Doi. II: Kochi Pref., Aki City, Ochiai, along the Suginokuma river. III: Tokushima Pref., Naka-gun, Naka-cho, along the Senbon valley.

TABLE 2. Sample list.

No.	Species	Locality	Collector
1	*Arisaema sikokianum*	Kochi Pref. Nankoku City, Nebiki-Pass	HayH
2	*A. sikokianum*	Kochi Pref. Kami City, Iwakai	MuY, HA
3	*A. sikokianum*	Tokushima Pref. Naka-Gun, Naka-Cho, Senbondani Valley	HayH, MuY, MiY
4	*A. sikokianum*	Kochi Pref. Aki City, Ochiai, Suginokuma River	HayH
5	putative hybrid	Kochi Pref. Aki City, Doi	HamH
6	putative hybrid	Kochi Pref. Aki City, Ochiai, Suginokuma River	HamH
7	putative hybrid	Tokushima Pref. Naka-Gun, Naka-Cho, Senbondani Valley	HamH
8	*A. tosaense*	Tokushima Pref. Naka-Gun, Naka-Cho, Senbondani Valley	HayH, MuY, MiY
9	*A. tosaense*	Kochi Pref. Aki City, Ochiai, Suginokuma River	HayH
10	*A. tosaense*	Kochi Pref. Nankoku City, Nebiki-Pass	HayH
11	*A. tosaense*	Kochi Pref. Kami City, Monobe-Cho	HayH *et al.*

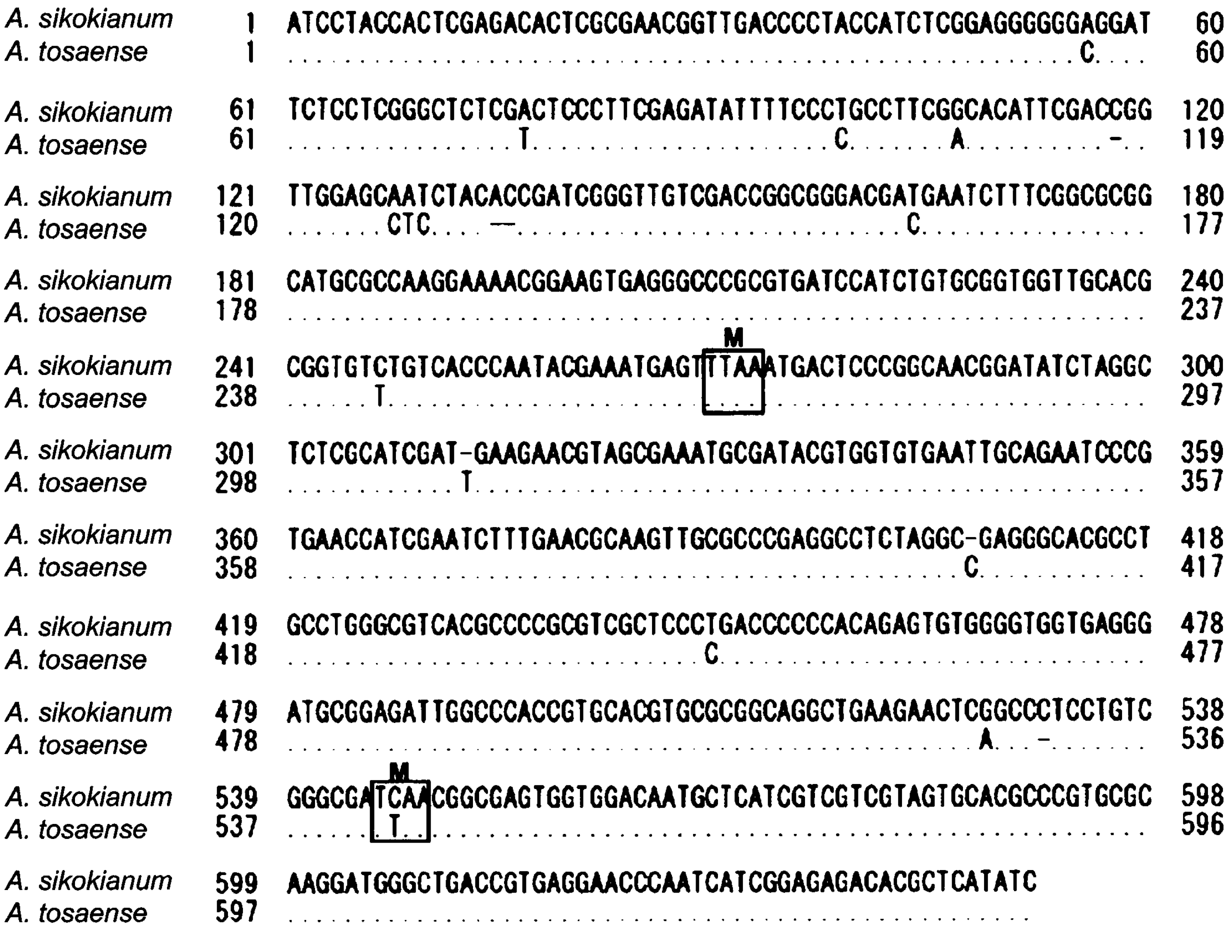
HayH: Hayakawa H. MuY: Muramatsu Y. HA: Hirata A. MiY: Minamiya Y. HamH: Hamachi H.

Genomic DNA Mini Kit (VIOGENE, Sunnyvale, USA), according to the manufacturer's protocol. We amplified the internal transcribed spacer (ITS) region from nrDNA and the *trnL* intron from cpDNA with primers designed by White *et al.* (1990) and Taberlet *et al.* (1991), respectively. The isolated DNA was amplified by PCR in a 50 μl reaction solution containing approximately 50 ng total DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 units *Taq* DNA polymerase (TaKaRa) and 0.5 μM of each primer. We used the following thermal cycle profile for amplification by the PCR Thermal Cycler Dice (TaKaRa): 1 min at 94°C, 2 min at 48°C, and 2 min at 72°C for 45 cycles, followed by 15 min of final extension at 72°C. We preliminarily sequenced the ITS region from the parental species to determine if different sequences could be used for verification of hybridization (Fig. 3). After amplification, the PCR products of the ITS region as well as the *trnL* intron were subjected to electrophoresis in 1% low-melting-temperature agarose gels to remove by-products and purify amplified products. We sequenced the purified PCR products using a BigDye Terminator ver. 3.1 (Applied BioSystems) and ABI Prism 3100 Genetic Analyzer (Applied BioSystems) according to the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification.

For the ITS region, we carried out PCR-RFLP analysis after checking the sequencing results and alignments. The result of the alignments indicated that an autapomorphic character of the nrDNA was the restriction site *Mse* I. After designating the restriction sites, the amplified products were digested by *Mse* I at 37°C for more than an hour. The digested DNAs were separated on 1.5% agarose gel and the size of each band was determined.

Results and Discussion

From the results of the molecular analysis we determined that one putative hybrid individual occurred at each locality. Morphologically, of the putative hybrids one has a purple spathe and a white, capitate appendage (Fig. 1C), which is similar to *Arisaema sikokianum*, but the leaf has nine leaflets, which is similar to the leaf of *A. tosaense* (located on Fig. 2I; Table 1). The second hybrid has a green spathe with an elongate tip and a cylindrical appendage, which is similar to that of *A. tosaense*, but the leaflets have the silhouette, width, serrations and non-elongate tip, similar to *A. sikokianum* (located on Fig. 2II). The third hybrid has a green spathe with an elongate tip (Fig. 1D) similar to *A. tosaense*, but has a capitate ap-



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A. sikokianum    1  ATCCTACCACTCGAGACACTCGCGAACGGTTGACCCCTACCATCTCGGAGGGGGGAGGAT   60
A. tosaense      1  .......................................................C....   60

A. sikokianum   61  TCTCCTCGGGCTCTCGACTCCCTTCGAGATATTTTCCCTGCCTTCGGCACATTCGACCGG  120
A. tosaense     61  ................T.....................C.......A..........-..  119

A. sikokianum  121  TTGGAGCAATCTACACCGATCGGGTTGTCGACCGGCGGGACGATGAATCTTTCGGCGCGG  180
A. tosaense    120  .......CTC....—...........................C................  177

A. sikokianum  181  CATGCGCCAAGGAAAACGGAAGTGAGGGCCCGCGTGATCCATCTGTGCGGTGGTTGCACG  240
A. tosaense    178  ............................................................  237

                                                   M
A. sikokianum  241  CGGTGTCTGTCACCCAATACGAAATGAGTTTAAATGACTCCCGGCAACGGATATCTAGGC  300
A. tosaense    238  ......T.....................................................  297

A. sikokianum  301  TCTCGCATCGAT-GAAGAACGTAGCGAAATGCGATACGTGGTGTGAATTGCAGAATCCCG  359
A. tosaense    298  ............T...............................................  357

A. sikokianum  360  TGAACCATCGAATCTTTGAACGCAAGTTGCGCCCGAGGCCTCTAGGC-GAGGGCACGCCT  418
A. tosaense    358  ...............................................C............  417

A. sikokianum  419  GCCTGGGCGTCACGCCCCGCGTCGCTCCCTGACCCCCCACAGAGTGTGGGGTGGTGAGGG  478
A. tosaense    418  .............................C..............................  477

A. sikokianum  479  ATGCGGAGATTGGCCCACCGTGCACGTGCGCGGCAGGCTGAAGAACTCGGCCCTCCTGTC  538
A. tosaense    478  ................................................A...-.......  536

                           M
A. sikokianum  539  GGGCGATCAACGGCGAGTGGTGGACAATGCTCATCGTCGTCGTAGTGCACGCCCGTGCGC  598
A. tosaense    537  .......T....................................................  596

A. sikokianum  599  AAGGATGGGCTGACCGTGAGGAACCCAATCATCGGAGAGACACGCTCATATC
A. tosaense    597  ....................................................
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FIG. 3. Expected restriction sites of *Mse* I for molecular characteristics of ITS regions by PCR-RFLP. M: restriction site.

pendage, which is similar to *A. sikokianum* (located on Fig. 2III). Among the putative hybrids, the shape and color of the spathe and appendage have characteristics of both species, but do not share the same morphological features.

The length of the ITS sequences of the nrDNA is 650bp in *Arisaema sikokianum* and 648 bp in *A. tosaense*. The sequences have been deposited in DDBJ/EMBL/GenBank International DNA databases (*A. sikokianum*: AB513176; *A. tosaense*: AB513177). Based on the sequence data, we found the amplified ITS region of *A. sikokianum* to have one *Mse* I site, while *A. tosaense* has two sites digested by this restriction enzyme (Fig. 3). We conducted PCR-RFLP to obtain further evidence of their hybrid nature. The digestion patterns of all samples of *A. sikokianum* and *A. tosaense* showed the expected patterns, and all putative hybrids showed the combined patterns of *A. sikokianum* and *A. tosaense* (Fig. 4). We therefore confirmed that the putative hybrids were truly hybrids between *A. sikokianum* and *A. tosaense*.

In the molecular phylogenetic analysis of cpDNA of *Arisaema*, Renner *et al.* (2004) indicated that there were no sequence differences between *A. sikokianum* and *A. tosaense* in the *trnL–F* intergenic spacer. Many regions of cpDNA have been reported to have nucleotide substitution rates that are sufficiently high to facilitate the elucidation of relationships among closely related species (e.g., Soltis & Soltis 1998). Of these, some studies have suggested that the *trnL* intron is an efficient region for detecting sequence differences among closely related species of various plant taxa (e.g., Fukuda *et al.* 2001, Yamashiro *et al.* 2004). We therefore determined partial sequences for this region for *A. sikokianum* and *A. to-*

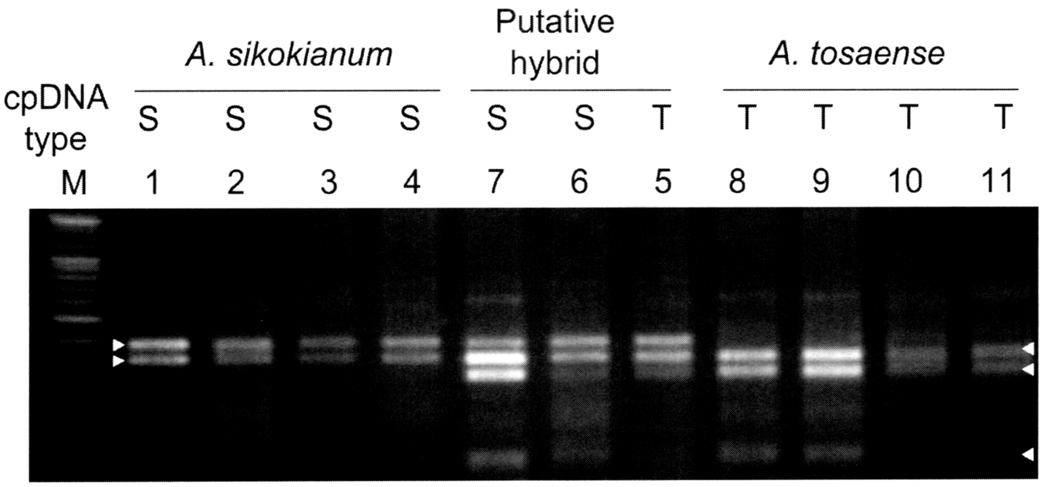

FIG. 4. PCR-RFLP profile of *Arisaema sikokianum*, *A. tosaense* and putative hybrids. Arrows indicate expected fragments of both *A. sikokianum* and *A. tosaense*. M: size marker. Sample numbers and cpDNA types corresponded to numbers and types in Table 2 and Table 3, respectively.

```
A. sikokianum    1  TGGTATGGAACCTACTAAGTGGTAACTTCCAAACTCAGAGAAACCCTGGAATAAAAAATG   60
A. tosaense      1  ............................................................   60

A. sikokianum   61  GGCAATCCTGAGCCAAATCCTTGTTTTTTTTTGAGAAAAAAGGATAGGTGCAGAGACTCG  120
A. tosaense     61  ............................................................  120

A. sikokianum  121  ATGGAAGCTGTTCTAACAAATGGAGTTGATTGCATTGCGTTGGTAGCTGGAATTCTTCTT  180
A. tosaense    121  ............................................................  180

A. sikokianum  181  TCTATCTAAATTACAGAAAAGATAACCCTATATACCTAATACGCACGTATATATACTGAC  240
A. tosaense    181  ............................................................  240

A. sikokianum  241  ATATCAAACGATTAATCACGACCCCAATCCATAATTATTATTTTTATTTATATTTTTATT  300
A. tosaense    241  ............................................................  300

A. sikokianum  301  TATAATTTATATAAATTTA-----------------ATATAATATATATAATAAATTAAA  343
A. tosaense    301  ...................TAATTTATATAAATTTA........................  360

A. sikokianum  344  TTATAATATATAATTATAATATATAAAATTATAATATATAATATATATTAAGTTAAATAT  403
A. tosaense    361  ............................................................  420

A. sikokianum  404  AATAAATATAATATAAATATAATATTAAATATAATATATATAATAAA
A. tosaense    421  ...............................................
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FIG. 5. Results of alignment of *trnL* intron sequences of *Arisaema sikokianum* and *A. tosaense*.

saense and found the length to be 450 bp in *A. sikokianum* and 467 bp in *A. tosaense*, indicating a 17 bp-insertion or deletion (indel) in the sequence of *A. sikokianum* (Fig. 5); the sequences have been deposited in DDBJ/EMBL/GenBank International DNA databases (*A. sikokianum*: AB513178; *A. tosaense*: AB513179). Moreover, this deletion was conserved in all individuals we examined of *A. sikokianum*. The *trnL* intron region is therefore a good molecular marker for distinguishing *A. sikokianum* from other species of *Arisaema* in Shikoku (data not shown). In the putative hybrids, the sequence results from the cpDNA analyses were identical to either *A. sikokia-*

TABLE 3. Genotype of sample used in this study.

Species	Locality	nrDNA	cpDNA
Arisaema sikokianum		S	S
Putative hybrid	I	S/T	T
	II	S/T	S
	III	S/T	S
A. tosaense		T	T

S: *A. sikokianum* type. T: *A. tosaense* type.

num or *A. tosaense* (Table 3), indicating that the hybrids had different maternal parents and that hybridization has occurred bidirectionally.

In considering the morphological diversity and the bidirectionally pollination patterns in the hybrids, backcrossing and/or introgression may be suspected. More detailed investigations are needed to determine these possibilities.

Fifteen putative natural hybrids have been reported in *Arisaema* (Murata 1962, Serizawa 1975, Ohno & Tsukada 1986, Murata & Ohno 1989, Kobayashi *et al.* 2005). Moreover, in Shikoku, Maki & Murata (2001) indicated that *Arisaema ehimense* J. Murata & Ohno is of hybrid origin between *A. serratum* (Thunb.) Schott and *A. tosaense* based on allozyme analysis. Those reports along with our findings suggest that hybrid speciation and/or reticulate evolution might have been occurred among *A. tosaense*, *A. sikokianum* and *A. serratum* in Shikoku.

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References

Fukuda, T., J. Yokoyama & H. Ohashi. 2001. Phylogeny and biogeography in *Lycium* (Solanaceae): Inferences from chloroplast DNA sequences. Mol. Phylogenet. Evol. 19: 246–258.

Kobayashi, S. 2009. Araceae. *In*: Kochi Prefecture & Makino Foundation of Kochi Prefecture (eds.), Flora of Kochi, pp. 604–607. Koubun Printing Office, Kochi. (In Japanese)

Kobayashi, T., J. Murata & K. Watanabe. 2005. Two new putative natural hybrids in Japanese *Arisaema* (Araceae). Acta Phytotax. Geobot. 56(1): 105–110.

Maki, M. & J. Murata. 2001. Allozyme analysis of the hybrid origin of *Arisaema ehimense* (Araceae). Heredity 86: 87–93.

Murata, G. 1962. Taxonomical note 7. Acta Phytotax. Geobot. 19: 67–72.

Murata, J. & J. Ohno. 1989. *Arisaema ehimense* J. Murata et Ohno (Araceae), a new species from Shikoku, Japan, of putative hybrid origin. J. Jap. Bot. 64: 341–351.

Ohashi, H. 1982. Araceae. *In*: Satake, Y., J. Ohwi, S. Kitamura, S. Watari & T. Tominari (eds.), Wild flowers of Japan vol. I, pp. 127–139. Heibonsha, Tokyo. (in Japanese)

Ohno, J. & M. Tsukada. 1986. *Arisaema yamatense* × *sikokianum*, a new natural hybrid of *Arisaema* (Araceae) in Japan. J. Jap. Bot. 61: 89–90. (in Japanese)

Renner, S. S., L. B. Zhang & J. Murata. 2004. A chloroplast phylogeny of *Arisaema* (Araceae) illustrates tertiary floristic links between Asia, North America and East Africa. Amer. J. Bot. 91: 881–888.

Serizawa, S. 1975. *Arisaema* of Tokyo-to. Tokyo-to no shizen No. 3: 1–7. (in Japanese)

Soltis, D. E. & P. S. Soltis. 1998. Choosing an approach and an appropriate gene for phylogenetic analysis. *In*: Soltis, D. E., P. S. Soltis & J. J. Doyle (eds.), Molecular Systematics of Plants II, pp. 1–42. Kluwer Academic Publishers, Boston.

Taberlet, P., L. Gielly, G. Pautou & J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17: 1105–1109.

White, T. J., T. Bruns, S. Lee & J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M., D. Gelfand, J. Sninsky & T. J. White (eds.), PCR Protocols: A Guide to Methods and Application, pp. 315–322. Academic Press, San Diego.

Yamashiro, T., T. Fukuda, J. Yokoyama & M. Maki. 2004. Molecular phylogeny of *Vincetoxicum* (Apocynaceae-Asclepidioideae) based on the nucleotide sequences of cpDNA and nrDNA. Mol. Phylogenet. Evol. 31: 689–700.

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